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CRISPR RNA-guided activation of endogenous human genes

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Abstract

Catalytically inactive CRISPR-associated 9 nuclease (dCas9) can be directed by short guide RNAs (gRNAs) to repress endogenous genes in bacteria and human cells. Here we show that a dCas9-VP64 transcriptional activation domain fusion protein can be directed by single or multiple gRNAs to increase expression of specific endogenous human genes. These results provide an important proof-of-principle that CRISPR-Cas systems can be used to target heterologous effector domains in human cells.

Clustered regularly interspaced short palindromic repeats (CRISPR) systems from bacteria¹ have enabled the development of RNA-guided nuclease technology for targeted genome editing^{2–7}. The *S. pyogenes* CRISPR-associated 9 nuclease (hereafter Cas9) can be directed by a ~100 nt single guide RNA (sgRNA) to a target genomic DNA sequence that is complementary to the first 20 nts of the sgRNA and flanked by a protospacer adjacent motif (PAM) sequence of the form NGG^{4,5}. RNA-mediated recruitment of a catalytically inactive mutant form of Cas9 (referred to as dCas9) can induce repression of endogenous genes in bacterial and human cells^{8,9}. In addition, fusions of dCas9 to effector domains have been used to activate reporter genes in *E. coli* and human cells^{9,10} and to repress endogenous genes in human cells¹⁰. Here we sought to determine whether dCas9-based transcriptional activators could regulate endogenous gene expression in human cells using single and multiple sgRNAs.

To do this, we constructed a dCas9-VP64 fusion protein consisting of the synthetic VP64 activation domain¹¹ linked to the carboxy-terminus of the catalytically inactivated dCas9 protein (Fig. 1a; Online Methods) and tested the ability of this fusion to activate expression of the human *VEGFA* gene. dCas9-VP64 protein was targeted to specific sites in the

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Author Contributions

M.L.M. and J.K.J. conceived of the study, designed the experiments, and wrote the manuscript. M.L.M., S.J.L., V.M.C., Y.F., Q.H.H. performed experiments.

Conflict of Interest Statement

M.L.M. and J.K.J. are inventors on a patent application describing the dCas9-VP64 fusion protein and its use to activate gene expression. J.K.J. has a financial interest in Transposagen Biopharmaceuticals. J.K.J.'s interests were reviewed and are managed by Massachusetts General Hospital and Partners HealthCare in accordance with their conflict of interest policies.

genome of human cells using 16 pre-validated sgRNAs (Supplementary Results and Supplementary Table 1) designed to bind sequences within three DNase I hyper-sensitive sites (HSSs) located upstream, downstream or at the *VEGFA* gene transcription start site (Fig. 1b). 15 of these 16 sgRNAs (designated V1-V16) induced significant increases in VEGFA protein expression when co-expressed with dCas9-VP64 in human 293 cells (Fig. 1c). Expression of a codon optimized dCas9-VP64 did not enhance the level of VEGFA activation observed (Supplementary Fig. 1). Expression of each of the 16 sgRNAs alone, dCas9-VP64 alone, or dCas9-VP64 together with an “off-target” sgRNA designed to bind an *EGFP* reporter gene sequence all failed to induce elevated VEGFA expression (Fig. 1c). In addition, experiments with a subset of three sgRNAs demonstrate that activation depends on the presence of the VP64 domain in the dCas9-VP64 fusion (Supplementary Results).

We next tested whether RNA-guided activators could also induce expression of the human *NTF3* gene. To do this, we used six sgRNAs targeted to sequences within a predicted DNase HSS in the human *NTF3* promoter and codon-optimized dCas9-VP64 (Fig. 1d; Methods). All six sgRNAs induced significant increases in *NTF3* transcript levels (Fig. 1e) with mean levels of activated expression varying over a four-fold range. Experiments with a subset of three sgRNAs showed that activation depends on the VP64 domain (Supplementary Results). Decreasing the amounts of sgRNA and dCas9-VP64 expression plasmids transfected resulted in less activation of the *NTF3* gene (Fig. 1e), demonstrating a dose-dependent effect.

We envisioned that expression of multiple guide RNAs in a single cell might enable synergistic activation of endogenous gene targets as recently described for TALE-based activators^{12,13}, thereby enabling combinatorial control of endogenous gene expression over a wide dynamic range. Co-expression of dCas9-VP64 with four sgRNAs (V1, V2, V3 and V4) as well as with subsets of three of these four sgRNAs induced synergistic activation of VEGFA protein expression (i.e.—levels of expression significantly greater than the expected additive effects of individual activators) (Fig. 2a). Similarly, co-expression of dCas9-VP64 with three sgRNAs (N4, N5, and N6) and all possible pairs of these sgRNAs led to large increases in *NTF3* expression with three of the four combinations yielding a mean *NTF3* mRNA level greater than the expected additive effects of the individual activators (Fig. 2b). Because synergy is mediated by binding of multiple activators to a single promoter, our results strongly suggest that multiple sgRNA/dCas9-VP64 complexes can function efficiently together in a single cell.

Our experiments define an RNA-guided activator platform that can be easily used to induce expression of endogenous genes in human cells. 21 of 22 sgRNAs tested successfully induced significant gene activation at the *VEGFA* and *NTF3* gene promoters (although these activators showed substantial variability in activity even when targeted to the same locus (Supplementary Discussion)). However, the magnitude of *VEGFA* and *NTF3* gene expression increases induced by our dCas9-VP64 activators generally appeared to be lower than what we previously observed using VP64-based TALE activators at these same endogenous genes.¹² Larger-scale testing will be needed to more definitively determine the relative success rates and potencies of RNA-guided activators and TALE-based activators in human cells.

An important priority for future experiments will be to define the extent of off-target effects induced by RNA-guided activators. sgRNA-guided Cas9 nuclease can induce off-target mutations at sites that differ by as many as five positions from the on-target sequence in human cells but *bona fide* off-target sites can be difficult to predict¹⁴. However, although nucleases can in principle alter the DNA sequence of any off-target site, a transcriptional activator may not always induce changes in gene expression when it binds to an off-target

site. Consistent with this, we note that a recent study found minimal, if any, changes in gene expression changes in human cells (as measured by RNA-Seq) induced by an RNA-guided dCas9-KRAB repression domain fusion¹⁰. Additional studies will be needed to determine whether this high degree of specificity is a general property of RNA-guided dCas9 fusions.

In summary, our results (and those recently reported by others¹⁰) provide proof-of-principle that heterologous effector domains can be fused to dCas9 without disrupting its ability to complex and function with sgRNAs in mammalian cells. These findings open the door to fusion of dCas9 to other novel effector domains (e.g., histone modifying enzymes, modifiers of DNA methylation). The simplicity of sgRNA-mediated targeting together with the ability to recruit dCas9 fusions in multiplex fashion should enable important applications of these tools for biological research and synthetic biology.

Online Methods

Construction of sgRNA expression plasmids

Pairs of DNA oligonucleotides encoding the variable 20 nt sgRNA targeting sequences were annealed together to generate short double-strand DNA fragments with 4bp overhangs (Supplementary Table 2). These fragments were ligated into BsmBI-digested plasmid pMLM3636 to yield DNA encoding a chimeric +103 single-chain guide RNA^{3,5} under the expression of a human U6 promoter. The pMLM3636 plasmid and its full DNA sequence are available from Addgene (<http://www.addgene.org/crispr-cas>).

Construction of dCas-VP64 expression plasmids

DNA encoding the Cas9 nuclease harboring inactivating D10A/H840A mutations (dCas9) was amplified by PCR from plasmid pMJ841 (Addgene plasmid #39318) using primers that add a T7 promoter site 5' to the start codon and a nuclear localization signal at the carboxy-terminal end of the Cas9 coding sequences and cloned into a plasmid containing a CMV promoter as previously described³ to yield plasmid pMLM3629. Oligonucleotides encoding a triple FLAG epitope were annealed and cloned into XhoI and PstI sites in plasmid pMLM3629 to generate plasmid pMLM3647 expressing dCas9 with a C-terminal flag FLAG tag. DNA sequence encoding a Gly₄Ser linker followed by the synthetic VP64 activation domain was introduced downstream of the FLAG-tagged dCas9 in plasmid pMLM3647 to yield plasmid pSL690. The D10A/H840A mutations were also introduced by QuikChange site-directed mutagenesis (Agilent) into plasmid pJDS246, which encodes a FLAG-tagged Cas9 sequence that has been codon optimized for expression in human cells, to yield plasmid pMLM3668. DNA sequence encoding the Gly₄Ser linker and the VP64 activation domain were then cloned into pMLM3668 to yield a codon-optimized dCas9-VP64 expression vector named pMLM3705. The full amino acid sequence of the dCas9-VP64 protein is shown in Supplementary Figure 6.

Cell culture and transfection

Human Flp-In T-Rex HEK293 cells were obtained from Life Technologies and cultured and transfected as previously described¹². Cells were tested every two weeks for mycoplasma contamination. Briefly, all transfections were performed in triplicate using Lipofectamine LTX (Life Technologies). For transfections involving regulation of *VEGFA*, 160,000 cells were seeded into 24 well plates the day before transfection. 250 ng of plasmid encoding *VEGFA*-targeted sgRNA, 250 ng of plasmid encoding dCas9-VP64 or recoded dCas9-VP64, and 30 ng pmaxGFP plasmid (Lonza) were co-transfected using 0.5 µl PLUS reagent and 2.65 µl Lipofectamine LTX. For synergy experiments, the amount of each sgRNA was kept constant such that all transfections contained 62.5 ng of each sgRNA and empty U6 promoter plasmid was used to fill up to a total of 250 ng. For transfections involving

regulation of *NTF3*, 350,000 cells were seeded in 12 well plates the day before transfection. 100, 250, or 500 ng of plasmid encoding *NTF3*-targeted sgRNA, 250ng or 500ng of plasmid encoding codon optimized dCas9-VP64, and 60 ng pmaxGFP plasmid (Lonza) were co-transfected using 1 μ l PLUS reagent and 2.1, 2.65, or 5.1 μ l Lipofectamine LTX, respectively. For synergy experiments, the amount of each sgRNA was kept constant such that all transfections contained 83.3 ng of each sgRNA and empty U6 promoter plasmid was used to fill up to a total of 500 ng.

Enzyme-Linked Immunoblot Assays of VEGFA protein

Culture medium of Flp-In T-Rex HEK293 cells transfected with plasmids encoding *VEGFA*-targeted sgRNA and dCas9-VP64 was harvested 40 hours post-transfection and VEGFA protein expression was measured by ELISA as previously described¹².

Quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

Total RNA from Flp-In T-Rex HEK293 cells transfected with plasmids encoding *NTF3*-targeted sgRNAs and codon-optimized dCas9-VP64 was isolated two days post-transfection using the PureLink RNA Mini Kit (Ambion) and treated with TurboDNA-Free (Ambion) according to manufacturer's instructions. *NTF3* mRNA levels were measured by quantitative RT-PCR using a Taqman assay as previously described¹²

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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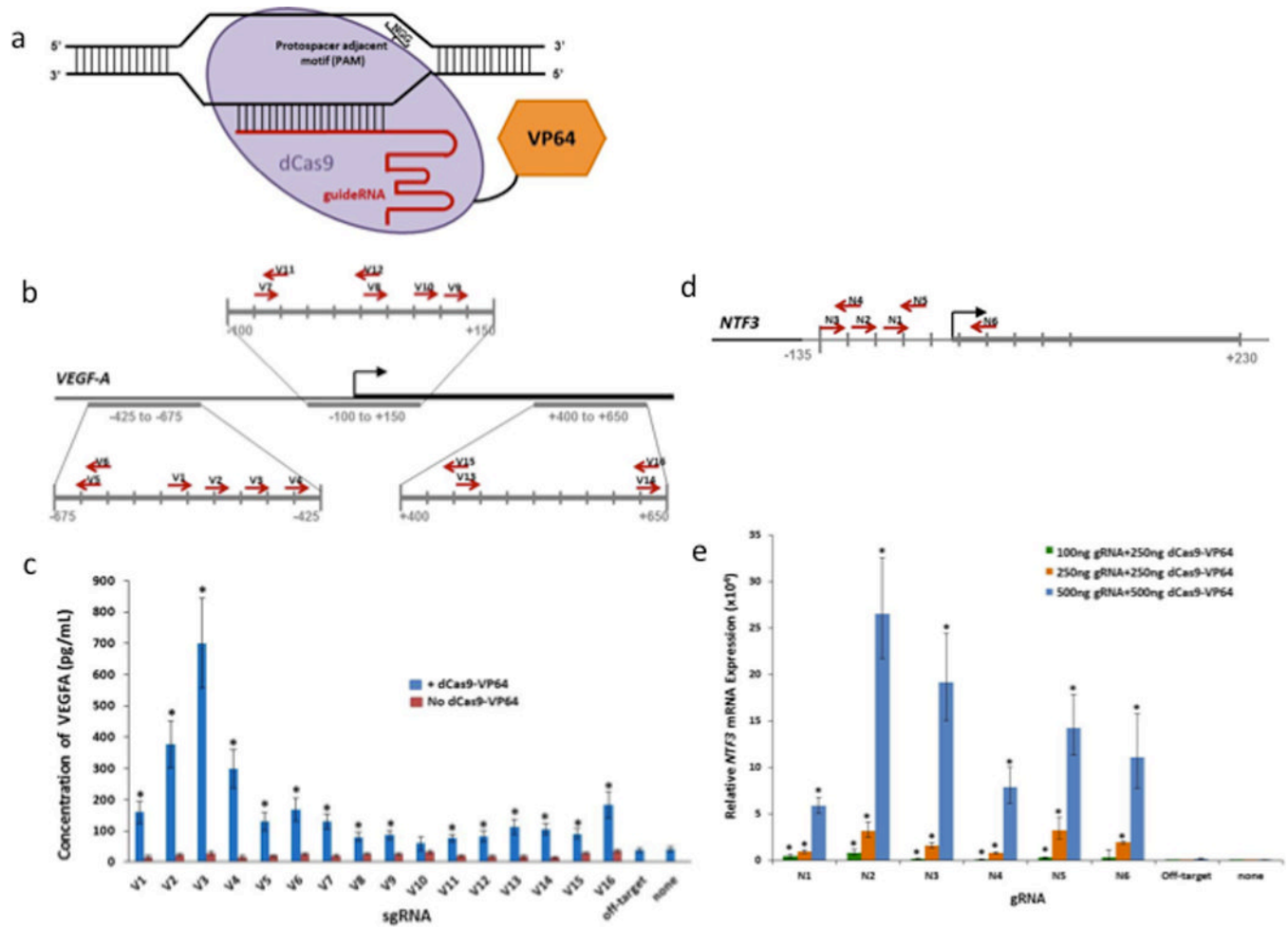


Figure 1. RNA-guided activation of endogenous human genes

(a) Schematic depicting recruitment of dCas9-VP64 fusion protein to a specific genomic target sequence by an sgRNA. (b) 16 sgRNAs targeted to the endogenous human *VEGFA* gene promoter. Red arrows represent the first 20 nts of the sgRNA pointing 5' to 3'. Grey bars indicate DNaseI hypersensitive sites previously defined in human 293 cells¹⁵, numbered relative to the transcription start site (black arrow). (c) Activation of VEGFA protein expression in 293 cells by various sgRNAs, each expressed with (blue bars) or without (red bars) dCas9-VP64. Asterisks indicate samples that are significantly elevated above the off-target control as determined by a paired, one-sided t-test ($n = 3$, $P < 0.05$); error bars represent s.e.m. (d) Six sgRNAs targeted to the endogenous human *NTF3* gene promoter represented as in (b). Grey line indicates region of potential open chromatin identified from the ENCODE DNaseI hypersensitivity track on the UCSC genome browser with the thicker part of the bar indicating the first transcribed exon. Numbering shown is relative to the transcription start site (black arrow). (e) Activation of *NTF3* gene expression by RNA-guided dCas9-VP64 in 293 cells. Relative expression of *NTF3* mRNA is shown for 293 cells co-transfected with the indicated amounts of codon-optimized dCas9-VP64 and *NTF3*-targeted sgRNA expression plasmids. Asterisks indicate samples that are significantly greater than the off-target sgRNA control as determined by a paired, one-sided t-test ($n = 3$, $P < 0.05$); error bars represent s.e.m.

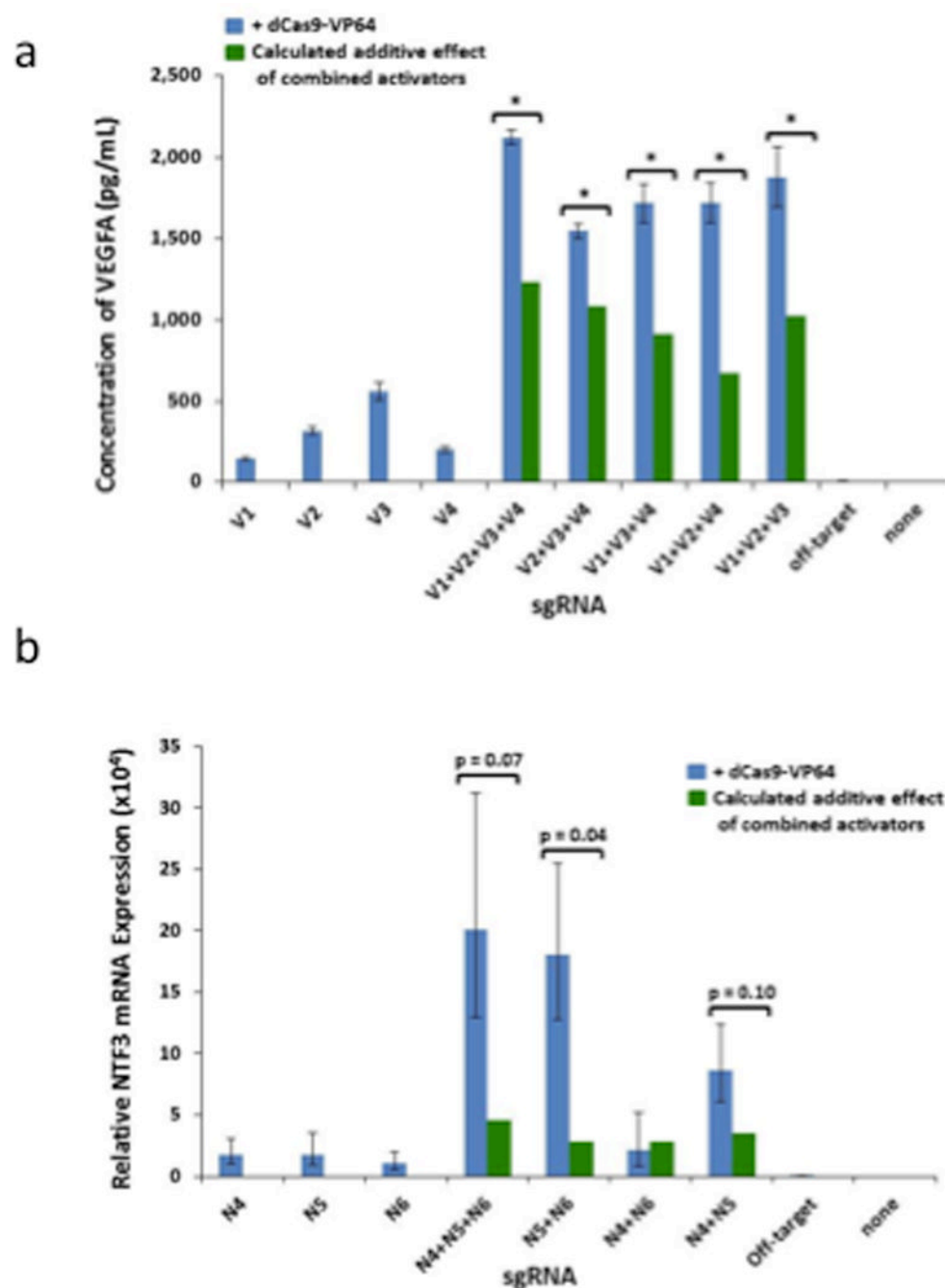


Figure 2. Synergistic activation of the endogenous human *VEGFA* and *NTF3* genes by RNA-guided activators

(a) Multiplex sgRNA expression induces synergistic activation of *VEGFA* protein expression by dCas9-VP64 protein. The calculated sum of mean *VEGFA* protein expression induced by individual sgRNAs is shown for each combination as green bars. Asterisks indicate all combinations that were found to be significantly greater than the expected sum as determined by an analysis of variance (ANOVA) ($n = 3$, $P < 0.05$). Error bars represent standard errors of the mean. (b) Multiplex sgRNA expression induces increased activation of *NTF3* mRNA expression by dCas9-VP64 protein. Relative expression of *NTF3* mRNA is shown for 293 cells in which the indicated individual or combinations of sgRNAs were co-

expressed with dCas9-VP64. The calculated sum of mean *NTF3* mRNA expression induced by individual sgRNAs is shown for each combination as green bars. *P* values were determined by a paired, one-sided T-test ($n = 3$). Error bars represent standard errors of the mean.